Supplementary Information

Two-Photon FRET Pair based on Coumarin and DBD dyes

P. Wessig, N. Behrends, M. U. Kumke, U. Eisold, T. Meiling and C. Hille

General information:

Syntheses were performed under nitrogen atmosphere using standard Schlenk techniques. NMR spectra were recorded on a Bruker Avance 300 MHz and a Bruker Avance III 600 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) referenced to the solvent (^{1}H NMR: $CDCl_3$ = 7.27 ppm, MeOD-d_4 = 3.31 ppm and DMSO-d_6 = 2.50 ppm, acetone-d_6 = 2.05 ppm; ^{13}C NMR: $CDCl_3$ = 77.00 ppm, MeOD-d_4 = 49.15 ppm, acetone-d_6 = 29.92 ppm). Abbreviations to denote the multiplicity of a particular signal are: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet. High resolution mass spectra (HRMS) were recorded on a spectrometer using quadrupole. Melting points were determined in an Elektrothermal 9100 melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin Elmer FTIR spectrometer Spectrum 2 and a Perkin Elmer IR-881 spectrometer. UV/VIS spectra were recorded on a Jasco V-630 spectrometer. Fluorescence spectra were measured with a Horiba Jobin Yvon Fluoromax 4 and quantum yields with a Hamamatsu Absolute PL Quantum Yield Measurement System C9920. Flash chromatography was performed on silica gel 60 (40 - 63 µm). All reactions were monitored by thin layer chromatography (TLC). All reagents and solvents were purchased from commercial sources and purified depending on purity before used.

Spectroscopic data:

Table 1. Summary of spectroscopic data of compounds **2**, **4**, **8**, **10a**, and **10b**

Compound	Solvent	λ_{abs} / nm	λ_{em} / nm	τ_F / ns	ε / M^{-1} cm^{-1}	Φ_F	σ_2 / GM[a]
2	ACN	340	410	0.8[b]	13355	0.10	0.01 ± 26%
	DCM	342	400	0.8[b]	10425	0.11	0.01 ± 38%
4	ACN	349	429	1.6[b]	15168	0.22	0.41 ± 10%
	DCM	351	424	2.1[b]	15503	0.36	0.26 ± 06%
8	ACN	426[1]	545	21.4[c]	5683	0.41	0.20 ± 18%
	DCM	439[1]	548	24.8[c]	4300	0.35	0.16 ± 03%
10a	ACN	340, 431	559	22.7[d]	-	-	-
	DCM	340, 435	550	26.5[d]	-	-	-
10b	ACN	350, 430	559	22.2[d]	-	-	-
	DCM	353, 436	550	25.5[d]	-	-	-

[a]excited at 780 nm, GM = 10^{-50} cm^4 s $photon^{-1}$ (triple measurement); [b]fluorescence lifetime was detected at 420 nm (λ_{exc} = 375 nm); [d]fluorescence lifetime was detected at 570 nm (λ_{exc} = 440 nm); [e]fluorescence lifetime was detected at 559 nm (ACN), 550 nm (DCM) (λ_{exc} = 372 nm)

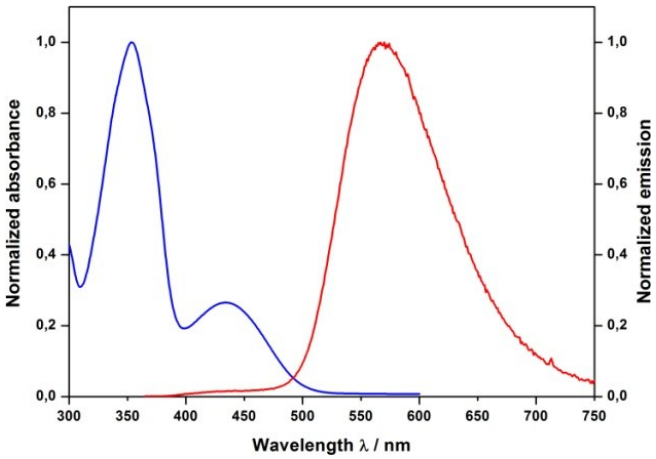

Figure SI-1 Absorption (blue) and emission (red) spectra of FRET pair **10b** in DMSO (λ_{exc} = 355 nm).

Calculation of the Förster distance R_0

The Förster distances R_0 were calculated according to the following equations:

$$R_0 = (8.79 \cdot 10^{-5} \cdot J \cdot q_D \cdot n^{-4} \cdot \kappa^2)^{1/6}$$

$$J = \int \varepsilon_A(\lambda) f_D(\lambda) \lambda^4 d\lambda \ / \ \int f_D(\lambda) d\lambda$$

J	normalized spectral overlap between donor emission (f_D) and acceptor absorbance (ε_A)
q_D	fluorescence quantum yield of the donor emission in the absence of the acceptor
n	refractive index
κ^2	dipole orientation factor

R_0 values were calculated for DMSO (n = 1.4793) and assuming freely rotating chromophors (κ^2 = 2/3)

Compound	q_D	R_0 / nm
10a	0.17	2.3
10b	0.68	2.9

Calculation of two-photon absorption cross section:

The two-photon excitation (2PE) measurements were carried out using a FLS9020 fluorescence spectrometer (Edinburgh Instruments Ltd., Livingston, UK) equipped with a R928P photomultiplier (Hamamatsu, Japan) to detect optical signals. Samples were measured in 1.0 x 1.0 cm quartz cuvettes in a 90° setup with direct excitation from a femtosecond (< 100 fs) titan-sapphire-laser (Tsunami HP, Spectra Physics, Santa Clara, CA, USA) with an 80 MHz repetition rate. The measurements were carried out at the wavelength of 780 nm. The pulse spectrum (FWHM 10 nm) was monitored throughout the measurements by a computer-controlled Avaspec-spectrometer (Avantes BV, Apeldoorn, NL). An achromatic NIR-lens (Thorlabs GmbH, München, Germany) to focus the laser

beam was integrated into the experimental setup, along with a neutral density (ND) filter for average laser power adjustment (200 mW to 500 mW). The laser power was monitored by an optical power meter right before starting the measurement. Spectral resolution was set to 1 nm; differences in signal intensity were compensated by adjusting the integration times (0.1 s to 2 s). The dye concentrations were varied between $2.4x10^{-5}$ M - $1.8x10^{-4}$ M for optimal signal intensities, depending on the dye and solvent used. Each sample were measured three times.

Two photon excited FLIM microscopy:

Two-photon excited fluorescence lifetime imaging microscopy (2PE-FLIM) has been performed as previously described.[2] Briefly, freshly prepared salivary gland lobes from American cockroaches were incubated with 2 µM 2PE-FRET pair **10b** for 15 min in physiological saline. 2PE-FLIM images were recorded using a MicroTime 200 microscope system (PicoQuant, Berlin, Germany) coupled to a fs-fiber laser working at 780 nm and 50 MHz repetition rate (MenloSystems, Martinsried, Germany). Fluorescence was detected within the spectral range of 420-680 nm in the time-correlated single-photon counting mode.

Synthetic procedures:

Benzyl 2-(8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetate (2): To a solution of **1** (200 mg, 1.04 mmol) and benzyl prop-2ynoate (200 mg, 1.25 mmol, 1.2 eq.) in dry DCM (10 mL) were added DMAP (203 mg, 1.67 mmol, 1.6 eq.) under N_2 atmosphere at room temperature. After being stirred over night, the solvent was removed under reduced pressure. Purification of the residue by flash silica gel column chromatography (Hex/EE 3:1) afforded **2** (330 mg, 0.94 mmol, 90%) as a white crystalline solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.28 - 7.39 (m, 5H), 6.92 (s, 1H), 6.78 (s, 1H), 6.64 (t, ^3J=5.1 Hz, 1H), 6.16 (d, ^4J=1.1 Hz, 1H), 5.20 (s, 2H), 3.08 (d, ^3J=5.1 Hz, 2H), 2.35 (d, ^4J=1.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 167.4, 161.0, 152.3, 150.5, 150.4, 144.5, 135.1, 128.6, 128.5, 128.4, 113.9, 112.3, 109.4, 102.2, 98.4, 67.1, 40.0, 19.0; mp: 97 - 98°C; IR: 3439, 3088, 3059, 3031, 2945, 1724, 1630, 1582, 1375, 1500, 1452, 1419, 1395, 1344, 1309, 1268, 1221, 1193, 1144, 1118, 1048, 1029, 984, 923, 859, 838, 805, 747, 699 cm^{-1}; HRMS m/z: $[M]^+$ calcd for $C_{20}H_{16}O_6$: 352.0943, found: 352.0947.

2-(8-Methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetic acid (3a): A suspension of **2** (440 mg, 1.25 mmol) and Pd/C (10%, 13 mg, 0.01 mmol, 0.01 eq.) in dry THF (150 mL) was stirred under hydrogen atmosphere ($p(H_2)$ = 1 atm) at room temperature. After complete conversion of **2** monitored by TLC (24 h) the mixture was filtered over Celite® and washed with MeOH. The solvent was removed under reduced pressure and the residue was purified by flash silica gel column chromatography (DCM/MeOH 10:1) yielding **3a** (264 mg, 1.01 mmol, 80%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.25 (s, 1H), 7.06 (s, 1H), 6.66 (t, ^3J=5.1 Hz, 1 H), 6.22 (s, 1H), 3.05 (d, ^3J=5.1 Hz, 2H), 2.35 (d, ^4J=1.1 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 170.1, 161.0, 154.4, 151.2, 150.8, 145.1, 114.2, 112.1, 111.0, 103.7, 98.6, 19.498; mp: decomposition at 210°C; IR: 3458, 3057, 2965, 1736, 1676, 1620, 1576, 1496, 1451, 1403, 1373, 1351, 1269, 1245, 1210, 1183, 1157, 1055 cm^{-1}; HRMS m/z: $[M]^+$ calcd for $C_{13}H_{10}O_6$: 262.0472, found: 262.0462.

2-(7-Bromo-8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetic acid (3b): It was synthesized by following the procedure described above for **3a**, except purification. The residue was dissolved in DCM and **3b** was precipitated through adding 2 drops of oxalyl chloride. **3b** (227 mg, 0.67 mmol, 82%) yielded as a white solid. ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.24 (s, 1H), 6.89 (s, 1H), 6.70 (t,

^{3}J=4.9 Hz, 1H), 3.12 (d, ^{3}J=5.1 Hz, 2H), 2.59 (s, 3H); ^{13}C NMR (75 MHz, acetone-d_6) δ: 169.3, 157.2, 152.4, 151.9, 149.9, 146.2, 114.5, 111.6, 110.5, 104.0, 98.5, 40.1, 20.3; mp: decomposition at 200°C; IR: 3076, 2964, 2916, 1697, 1627, 1556, 1493, 1436, 1399, 1368, 1320, 1306, 1264, 1233, 1208, 1196, 1156, 1097, 1062, 1043, 1024, 972, 962, 882, 847, 838, 750, 675, 612 cm^{-1}; HRMS m/z: $[M]^+$ calcd for $C_{13}H_9BrO_6$: 339.9582, found: 339.9585.

Benzyl 2-(7-bromo-8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetate (4): To a solution of **2** (560 mg, 1.59 mmol) in dry ACN (10 mL) were added NBS (567 mg, 318 mmol, 2 eq.) and NaOAc (13 mg, 0.16 mmol, 0.1 eq.) under N_2 atmosphere at room temperature. The mixture was stirred till complete conversion of **2** monitored by TLC. The organic layer was washed with saturated NaCl solution and was extracted three times with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified by flash silica gel column chromatography (PE/EE 5:1) yielding **4** (680 mg, 1.58 mmol, quant.) as a white crystalline solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.34 (s, 5H), 6.96 (s, 1H), 6.77 (s, 1H), 6.66 (t, ^{3}J=5.0 Hz, 1H), 5.19 (s, 2H), 3.09 (d, ^{3}J=5.1 Hz, 2H), 2.54 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 167.8, 157.7, 151.3, 151.0, 149.3, 145.5, 135.4, 129.0, 128.9, 128.8, 114.1, 110.8, 110.2, 103.0, 98.6, 67.6, 40.5, 20.4; mp: 102-103°C; IR: 2953, 2918, 1739, 1713, 1630, 1556, 1488, 1445, 1440, 1379, 1317, 1256, 1208, 1165, 1148, 1108, 1062, 1024, 971, 853, 745, 734, 699, 615, 607, 524, 510, 495 cm^{-1}**;** HRMS m/z: $[M]^+$ calcd for $C_{20}H_{15}BrO_6$: 430.0052, found: 430.0056.

***tert*-Butyl (2-(2-(8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetamido)ethyl)carbamate (6a):** To a solution of **3a** (390 mg, 1.49 mmol) und *tert*-butyl(2-aminoethyl)carbamat (**5**, 262 mg, 1.64 mmol, 1.1 eq.) in dry DCM (70 mL) were added DCC (338 mg, 1.64 mmol, 1.1 eq.) and HOBt (221 mg, 1.64 mmol, 1.1 eq.) under N_2 atmosphere at room temperature. The reaction was monitored by TLC. After completion of the reaction (24 h), it was washed with 1N HCl and saturated NaCl solution, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified by flash silica gel column chromatography (DCM/MeOH 25:1) yielding **6b** (544 mg, 1.35 mmol, 90%) as a white crystalline solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.95 – 6.87 (m, 2H), 6.72 (s, 1H), 6.62 (t, ^{3}J=5.2 Hz, 1H), 6.10 - 6.15 (m, 1H), 5.13 - 5.01 (m, 1H, 14), 3.35 - 3.46 (m, 2H), 3.23 - 3.35 (m, 2H), 2.90 (d, ^{3}J=5.1 Hz, 2H), 2.32 – 2.33 (m, 3H), 1.43 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 167.1, 161.2, 157.0, 152.6, 150.5, 150.3, 144.6, 113.8, 112.1, 110.6, 102.1, 98.3, 79.8, 41.9, 41.0, 40.0, 28.3, 19.1; mp: 123 – 124.5°C; IR: 3624, 3330, 3096, 2740, 2683, 1837, 1732, 1682, 1636, 1583, 1561, 1527, 1491, 1450, 1387, 1365, 1347, 1324, 1264, 1203, 1169, 1142, 1098, 1074, 1047, 919, 856, 805, 784, 760 cm^{-1}; HRMS m/z: $[M+H]^+$ calcd for $C_{20}H_{25}N_2O_7$: 405.1662, found: 405.1659.

***tert*-Butyl (2-(2-(7-bromo-8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetamido)ethyl)carbamate (6b):** The title compound was synthesized by following the procedure described above for **6a** and was isolated as a white solid (95%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.95 (s, 1H), 6.93 – 6.85 (m, 1H), 6.73 (s, 1H), 6.65 (t, ^{3}J=5.3 Hz, 1H), 5.06 (t, ^{3}J=5.6 Hz, 1H), 3.49 - 3.35 (m, 2H), 3.35 - 3.21 (m, 2H), 2.91 (d, J=5.1 Hz, 2H), 2.52 (s, 3H), 1.55 - 1.34 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 167.0, 157.3, 151.0, 150.7, 148.7, 145.1, 113.6, 110.9, 110.1, 102.5, 98.1, 79.8, 41.9, 41.1, 40.0, 28.3, 20.0; mp: 182-183°C; IR: 3335, 3074, 2976, 2931, 2856, 1706, 1655, 1555, 1523, 1493, 1444, 1391, 1367, 1321, 1257, 1211, 1169, 1152, 1081, 1062, 1023, 973, 852, 749, 612 cm^{-1}; HRMS m/z: $[M]^+$ calcd for $C_{20}H_{23}BrN_2O_7$: 482.0689, found: 482.0678.

N-(2-Aminoethyl)-2-(8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetamide (7a): Compound **6a** was stirred in DCM/TFA (1:1; 5 mL) for 30 min. The mixture was concentrated under

reduced pressure ($1 \cdot 10^{-3}$ mbar) yielding **7a** (98 mg, 0.24 mmol, quant.) as a white resin. ^{1}H NMR (300 MHz, MeOD-d_4) δ: 7.13 (s, 1H), 6.84 (s, 1H), 6.65 (t, ^{3}J=5.2 Hz, 1H), 6.16 - 6.19 (m, 1H), 3.50 (t, ^{3}J=6.1 Hz, 2H), 3.07 (t, ^{3}J=6.0 Hz, 2H), 2.98 (d, ^{3}J=5.3 Hz, 2H), 2.38 – 2.41 (m, 3H); ^{13}C NMR (75 MHz, MeOD-d_4) δ: 171, 164, 156, 152, 152, 146, 115, 113, 112, 104, 99, 42, 41, 38, 19; IR: 2999, 2987, 2941, 1633, 1581, 1493, 1454, 1390, 1348, 1268, 1202, 1080, 1047, 1035, 927, 859, 836, 798, 721, 705 cm^{-1}; HRMS m/z: $[M]^{+\bullet}$ calcd for $C_{15}H_{16}N_2O_5$: 304.1059, found: 304.1068.

2-(2-(7-Bromo-8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetamido)ethan-1-aminium 2,2,2-trifluoroacetate (7b): The title compound was synthesized by following the procedure described above for **7a** and was isolated as a white solid (quant.). ^{1}H NMR (300 MHz, MeOD-d_4) δ: 7.26 (s, 1H), 6.92 (s, 1H), 6.68 (t, ^{3}J=5.1 Hz, 1H), 3.48 (t, ^{3}J=6.1 Hz, 2H), 3.05 (t, ^{3}J=6.1 Hz, 2H), 2.98 (d, ^{3}J=5.1 Hz, 2H), 2.61 (s, 3H); ^{13}C NMR (75 MHz, MeOD-d_4) δ: 170.9, 159.1, 153.7, 152.6, 150.3, 146.9, 115.1, 112.5, 110.5, 104.2, 98.9, 42.3, 40.8, 38.3, 20.4; IR: 3073, 2958, 2928, 2859, 1671, 1631, 1553, 1492, 1444, 1391, 1322, 1257, 1203, 1180, 1134, 1029, 976, 838, 80, 749, 722, 640, 612 cm^{-1}; HRMS m/z: $[M]^{+\bullet}$ calcd for $C_{15}H_{15}BrN_2O_5$: 382.0164, found: 382.0176.

2-(4,8-Dibutyrylbenzo[1,2-d:4,5-d']bis([1,3]dioxole)-2-yl)acetyl chloride (9): To a mixture of **8** (20 mg, 0.05 mmol) in dry DCM (5 mL) were added dropwise oxalyl chloride (47 µL, 0.55 mmol, 10 eq.) under N_2 atmosphere at room temperature. After stirring over night, the solvent was removed under reduced pressure and the orange residue was used for the next reaction without further purification.

2-(4,8-Dibutyrylbenzo[1,2-d:4,5-d']bis([1,3]dioxole)-2-yl)-N-(2-(2-(8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)acetamido)ethyl)acetamide (10a): To a solution of **7a** (23 mg, 0.05 mmol, 1.0 eq.) and DIPEA (96 µL, 0.55 mmol, 10 eq.) in dry DCM (2 mL) were added a solution a of **9** (21 mg, 0.05 mmol, 1.0 eq.) in dry DCM (3 mL) under N_2 atmosphere at room temperature. After stirring over night, the solvent was removed under reduced pressure and the residue was purified by flash silica gel column chromatography (DCM/MeOH 50:1) yielding **10a** (34 mg, 0.05 mmol, 94 %) as an orange solid. ^{1}H NMR (300MHz, $CDCl_3$) δ: 7.28 – 7.27 (m, 1H), 6.88 (s, 1H), 6.71 (s, 1H), 6.66 (t, ^{3}J=5.4 Hz, 1H), 6.58 (t, ^{3}J=5.3 Hz, 1H), 6.56 – 6.50 (m, 1H), 6.12 (d, ^{4}J=0.8 Hz, 1H), 6.09 (s, 2H), 3.60 – 3.38 (m, 4H), 3.01 (d, ^{3}J=5.5 Hz, 2H), 2.93 (d, ^{3}J=5.5 Hz, 2H), 2.87 – 2.81 (m, 4H), 2.31 (d, ^{4}J=0.9 Hz, 3H), 1.72 - 1.54 (m, 4H), 1.00 - 0.85 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 196.0, 167.7, 167.6, 167.0, 152.4, 150.4, 140.5, 140.4, 113.7, 112.1, 110.7, 110.0, 102.6, 102.1, 98.2, 45.7, 42.5, 41.7, 39.8, 39.5, 19.1, 17.1, 13.7; mp: 183°C; IR: 3379, 3291, 3085, 2928, 1729, 1683, 1649, 1582, 1557, 1492, 1439, 1394, 1394, 1282, 1267, 1143, 1109, 1090, 858 cm^{-1}; HRMS m/z: $[M]^{+}$ calcd for $C_{33}H_{34}N_2O_{12}$: 650.2112, found: 650.2134.

2-(7-Bromo-8-methyl-6-oxo-6H-[1,3]dioxolo[4,5-g]chromen-2-yl)-N-(2-(2-(4,8-dibutyrylbenzo[1,2-d:4,5-d']bis([1,3]dioxole)-2-yl)acetamido)ethyl)acetamide (10b): The title compound was synthesized by following the procedure described above for **10a** and was isolated as an orange solid (90%). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 6.94 (s, 1H), 6.75 (s, 1H), 6.70 (t, ^{3}J=5.5 Hz, 1H), 6.57 (t, ^{3}J=5.5 Hz, 1H), 6.37 (t, ^{3}J=5.8 Hz, 1H), 6.11 (dd, ^{3}J=1.1, 7.2 Hz, 2H), 3.55 - 3.45 (m, 4H), 3.03 (dd, ^{3}J=1.7, 5.5 Hz, 2H), 2.94 (d, ^{3}J=5.3 Hz, 3H), 2.85 (dt, ^{3}J=2.4, 7.2 Hz, 4H), 2.52 (s, 1H), 1.67 – 1.62 (m, 4H), 0.95 – 0.92 (m, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 195.5, 172.8, 156.6, 144.9, 140.5, 140.1, 103.3, 97.7, 44.9, 43.2, 41.6, 40.4, 23.4, 18.5, 13.9; mp: 213-214°C; IR: 3286, 3087, 2956, 2929, 2874, 1723, 1684, 1648, 1559, 1492, 1440, 1405, 1394, 1284, 1259, 1241, 1209, 1108, 1091, 936, 848, 748 cm^{-1}; HRMS m/z: $[M+H]^{+}$ calcd for $C_{33}H_{34}BrN_2O_{12}$: 729.1295, found: 729.1270.

NMR Spectra:

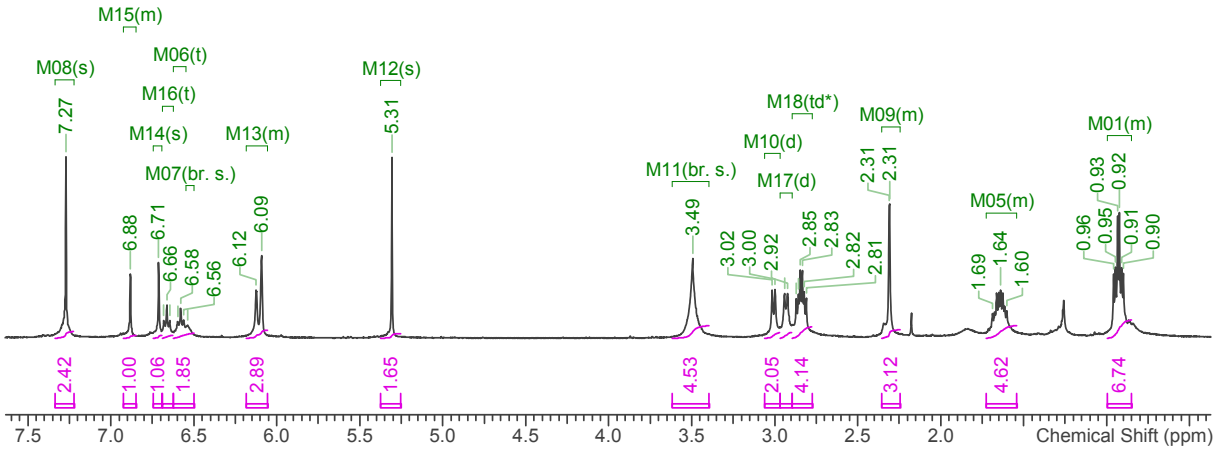

Figure SI-2. ^{1}H NMR of Compound **10a** in $CDCl_3$ (300 MHz)

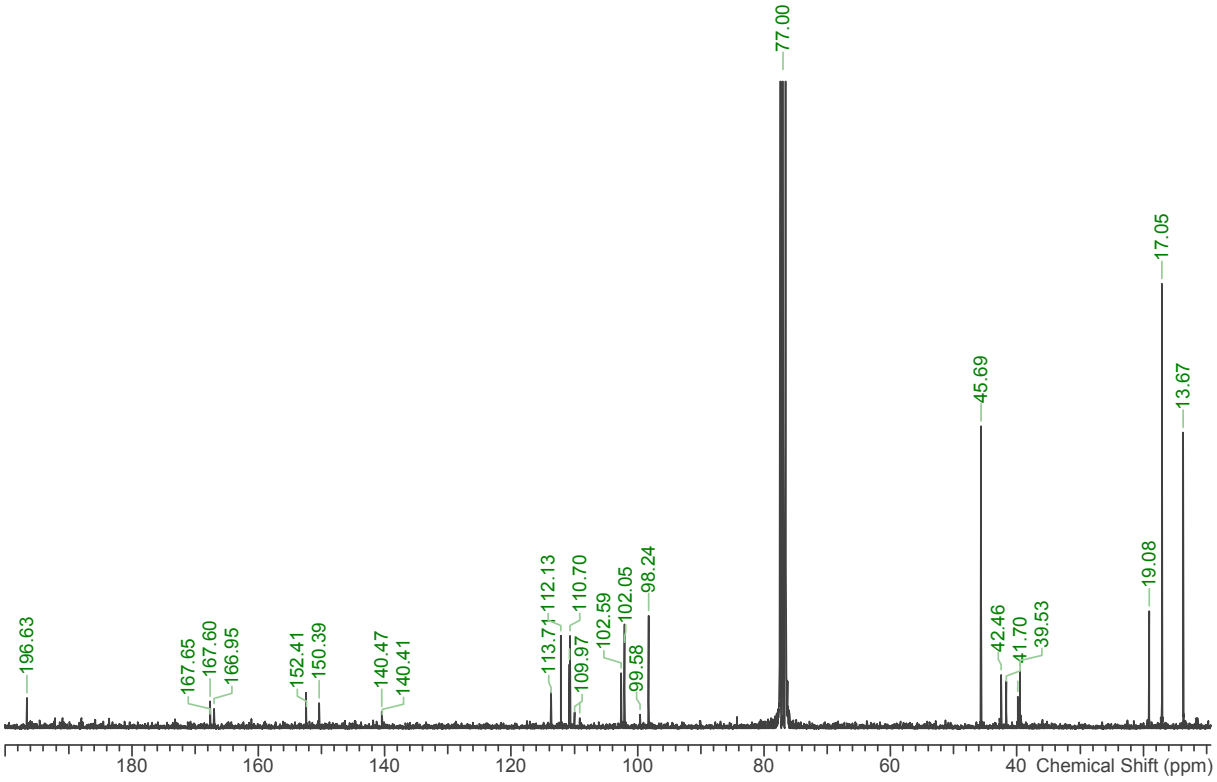

Figure SI-3. ^{13}C NMR of Compound **10a** in $CDCl_3$ (75 MHz)

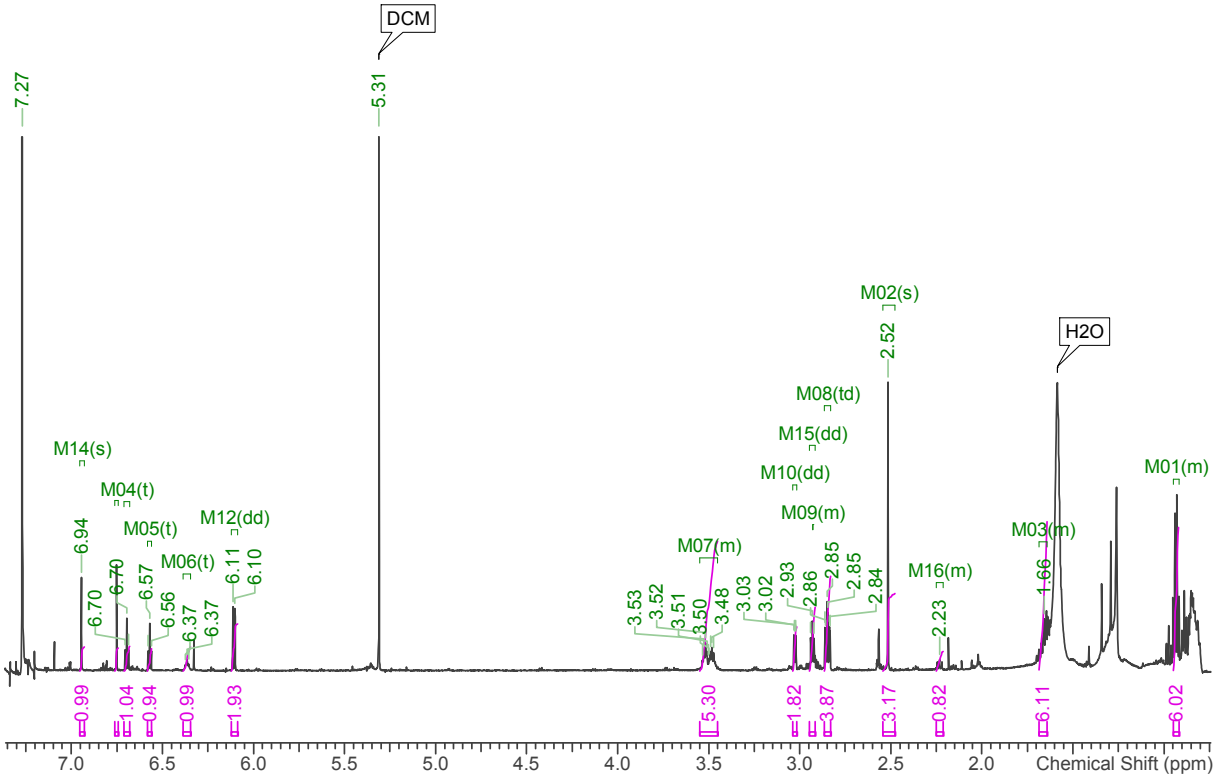

Figure SI-4. ^{1}H NMR of Compound **10b** in $CDCl_3$ (600MHz)

References:

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2 M. Lahn, C. Dosche, and C. Hille, *Am. J. Physiol. Cell Physiol.*, 2011, **300**, C1323.